THE ISOLATION OF FURTHER POLYPEPTIDES FROM THE THYLAKOID MEMBRANE, THEIR LOCALIZATION AND FUNCTION

F. KOENIG, G. H. SCHMID, A. RADUNZ, B. PINEAU and W. MENKE

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut) 5 Köln-Vogelsang, West Germany

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1. Introduction

In a previous publication we have reported on the preparation of polypeptide fractions of uniform molecular weights from the thylakoid membrane [1]. Their localization in the thylakoid membrane was achieved by means of specific antisera. The antisera affected photosynthetic electron transport specifically at different sites. In the present paper we report on the isolation of polypeptide fractions with the mol. wts. 11 000 (polypeptide 11 000), 17 000, 40 000 and 48 000 as well as on their localization in the thylakoid membrane and on their function.

2. Materials and methods

The general procedure of solubilization of stroma-freed chloroplasts from *Antirrhinum majus* [2] in sodium dodecyl sulfate-dodecyl sulfate) and mercaptoethanol-containing phosphate buffers as well as the isolation of the polypeptide fractions are described in the previous publication [1]. Additional details are already published [3]. In order to improve on the separation efficiency, the column length was increased from 90 to 370 cm. For the isolation of polypeptide 40 000, 0.01 M phosphate buffer pH 8.4, containing 0.08% dodecyl sulfate was used. In this alkaline buffer a much smaller portion of the thylakoid membrane was solubilized. The final purification step for polypeptide 11 000 comprised a gel permeation chromatography over Ultrogel LKB AcA 54 in 0.375 M Tris-HCl pH 8.8 containing 0.25% dodecyl sulfate and 0.1% mercaptoethanol.

The preparation of the antisera and the description of the Ouchterlony double diffusion tests in the presence of dodecyl sulfate have been described and referred to in the previous publication [1].

Partial reactions of photosynthetic electron transport are described earlier [4–6]. Light-triggered ATPase activity in tobacco chloroplasts was measured by following the hydrolyzation of [^{32}P]ATP according to Carmeli and Avron [7]. We analyzed for the remaining unhydrolized ATP and for the liberated [^{32}P]phosphate.

For technical reasons two types of chloroplast preparations from two plant species were used. A species specificity of the antisera was not observed.

3. Results and discussion

The antisera to all mentioned polypeptide fractions agglutinate stroma-freed chloroplasts. This implies that antibodies have been formed to native antigen determinants, and that these determinants are located in an accessible location in the outer surface of the thylakoid membrane.

The antiserum to polypeptide 11 000 inhibits the 2,6-dichlorophenol indophenol (DPiP) Hill reaction (fig.1) whereas the photosystem I mediated photoreduction of methylviologen with DPiP/ascorbate as the electron donors is unaffected (fig.2). Consequently, the inhibition site must be located in the region of light reaction II. The inhibition increases with the duration of illumination. The fact that the photoreduction of DPiP is unaffected when diphenylcarbazide is the electron donor instead of water clearly shows that the antiserum exerts its effect on the oxygen evolving side of photosystem II (fig.1).

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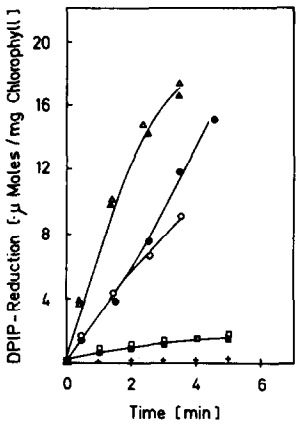

Fig.1. Hill reaction with DPiP as the electron acceptor in tobacco chloroplasts. (○) Reaction in the presence of antiserum to polypeptide 11 000; (●) reaction in the presence of control serum. (△,▲) Same reactions as before but in the presence of the uncoupler methylamine. (□) Photoreduction of DPiP in the presence of antiserum to polypeptide 11 000, with diphenylcarbazide as the electron donor instead of water; (■) reaction in the presence of control serum; (+) reaction with Tris-washed chloroplasts without serum and diphenylcarbazide addition. $2.4 \cdot 10^5$ erg·sec^{-1}·cm^{-2} of red light 580 nm $<\lambda<$ 750 nm.

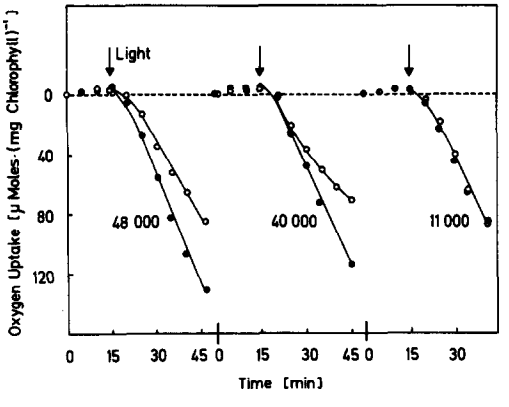

Fig.2. Inhibition of photosynthetic electron transport in tobacco chloroplasts by different antisera. Methylviologen Mehler reaction with DPiP/ascorbate as the electron donor in 20 000 erg·sec^{-1}·cm^{-2} of red light in the presence of 3-(3,4-dichlorophenyl)-1,1'-dimethylurea (DCMU), (○) reaction in the presence of antiserum; (●) reaction in the presence of control serum. The mol. wts. of the antigens used for the preparation of the antisera are indicated.

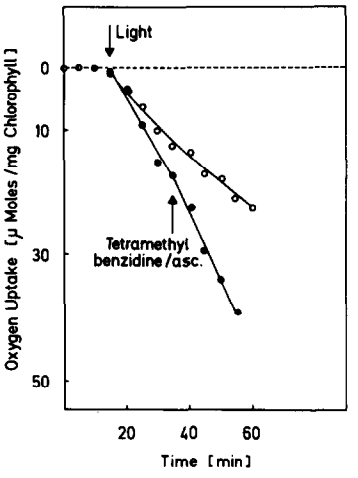

Fig.3. Photoreduction of methylviologen in tobacco chloroplasts with water as the electron donor. The arrow indicates the addition of tetramethyl benzidine acting as an electron donor to photosystem II even in the presence of 0_2-evolution. (○) Reaction in the presence of antiserum to polypeptide 11 000; (●) reaction in the presence of control serum. The reaction was measured in a Mehler type reaction as 0_2 uptake in 20 000 erg·sec^{-1}·cm^{-2} of red light.

In the presence of methylamine that is under an uncoupling condition no inhibition is observed (fig.1). In the Hill reaction with methylviologen as the electron acceptor the antiserum to polypeptide 11 000 also inhibits electron transport (fig.3). In this reaction water as the electron donor can be

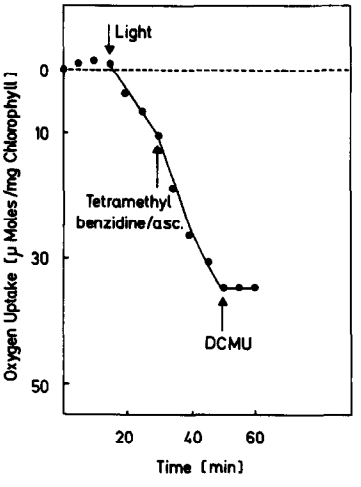

Fig.4. The same reaction as in fig.3 in order to show DCMU-sensitivity.

Table 1
Effect of antisera to polypeptides isolated from the lamellar system of *Antirrhinum majus* on photophosphorylation reactions in chloroplasts from *Nicotiana tabacum*

Antiserum to polypeptides of molecular weight	(μmoles [^{32}P]ATP formed·(mg Chlorophyll)$^{-1}$·h^{-1}) Non-cyclic $H_2O \rightarrow K_3Fe(CN)_6$	Non-cyclic $H_2O \rightarrow$ Methylviologen	Cyclic PMS-mediated
11 000	31.0 ± 0.8	13.8 ± 0.4	466 ± 3[a]
Control serum	33.5 ± 0.8	15.7 ± 0.4	408 ± 2
% Inhibition	7	12	0
40 000	5.1 ± 0.4	0-4.9	32.0 ± 0.5
Control serum	18.5 ± 0.8	13.4 ± 0.3	405 ± 4
% Inhibition	72.5	65–100	93
48 000	36.5 ± 0.9	11.9 ± 0.9	415 ± 2
Control serum	14.5 ± 0.7	16.8 ± 0.4	405 ± 3
% Inhibition	0	29	0
Control without serum addition	18.5 ± 0.8	9.4 ± 0.7	365 ± 3
Control without serum addition + DCMU	0.0	0.0	365 ± 3

The reaction was carried out at 15°C in 550 000 ergs·cm^{-2}·sec^{-1} white light. Chloroplasts for the reaction were prepared from *N. tabacum* var. John William's Broadleaf. [a]Are results from a different series of chloroplast preparations. All values are averages of at least 5 individual determinations. Variations are given as standard deviations.

substituted by tetramethyl benzidine (arrow fig.3). If this reaction is carried out in the presence of antiserum to polypeptide 11 000 it is not further inhibited, demonstrating that the observed inhibition is maximal. The maximal inhibition is 46%. The curve in fig.4 shows that the photoreduction of methylviologen with tetramethyl benzidine is sensitive to 3-(3,4-dichlorophenyl)-1,1′-dimethylurea (DCMU) which demonstrates that the reaction is mediated by photosystem II [12]. Non-cyclic photophosphorylation reactions in the system $H_2O \rightarrow K_3Fe(CN)_6$ or $H_2O \rightarrow$methylviologen are slightly inhibited by the antiserum whereas PMS*-mediated cyclic photophosphorylation which involves only light reaction I is not inhibited but appears slightly stimulated (table 1). Serological investigations by the Ouchterlony double diffusion test as well as end-group determinations have shown that the polypeptide fraction 11 000 consists of several components although gel electrophoresis in the dodecyl sulfate-Tris-glycine system according to Laemmli [8] shows one single narrow band.

*Phenazine methosulfate

The antiserum to polypeptide 40 000 inhibits photosynthetic electron transport on the photosystem I side. The methylviologen reduction with DPiP/ascorbate as the electron donors in the presence of DCMU is inhibited. The inhibition increases in the course of the reaction (fig.2). Typical photosystem II reactions such as the ferricyanide Hill reaction are not affected by the antiserum. The antiserum inhibits all tested photophosphorylation reactions almost completely (table 1) which suggests that the polypeptide 40 000 might correspond to the γ-component of the coupling factor described in the literature [9]. This is further suggested by the fact that the activity of the antiserum towards photophosphorylation is removed by preincubation of the antiserum with a coupling factor preparation (table 2). In contrast to the γ-component, described in the literature, the antiserum to polypeptide 40 000 as well as the antiserum to the earlier described polypeptide 62 000 enhance the light-triggered ATPase in tobacco chloroplasts (table 3). Thus, the antisera to polypeptide 40 000 and to the earlier described polypeptide 62 000 inhibit the photophosphorylation

Table 2
Effect of the antiserum to the polypeptide 40 000 on PMS-mediated cyclic photophosphorylation in chloroplasts from *Nicotiana tabacum*

Antiserum to polypeptide of mol. wt.	(μmoles [^{32}P]ATP-hydrolyzed·(mg Chlorophyll)$^{-1}$·h^{-1})
40 000	508
Control serum	598
40 000 preincubated with coupling factor CF_1	875[a]

Chloroplasts for the reaction were prepared from *N. tabacum* var. aurea Su/su^2. [a]The antiserum was incubated before use with 0.3 ml CF_1 corresponding to 0.3 mg protein. The reaction mixture was illuminated for 4 min at 15°C with 550 000 ergs·cm^{-2}·sec^{-1} white light. The antiserum was given in limiting amounts.

reaction by modifying the properties of the coupling factor. Obviously, the polypeptide fraction 40 000 contains the γ-component of the coupling factor for which the literature describes the apparent mol. wts. 37–38 000 [9,10].

The antiserum to polypeptide 48 000 inhibits the methylviologen Mehler reaction with DPiP/ascorbate as the electron donors. The inhibition is only observed if the DPiP concentration is sufficiently low, that is, if plastocyanin is included in the electron path [11]. Hence, the site of action of the antiserum is in the electron path between the two photosystems before plastocyanin. The inhibition site must be close to plastocyanin because PMS-mediated cyclic photophosphorylation and the non-cyclic photophosphorylation with ferricyanide as the electron acceptor are not inhibited. On the other hand, the photophosphorylation reaction in the system $H_2O \rightarrow$methylviologen is inhibited by nearly 30% (table 1). The inhibition site of this antiserum distinctly differs from that of the previously described antiserum to polypeptide 33 000 [1]. In the case of the antiserum to polypeptide 33 000 the PMS-mediated cyclic photophosphorylation is inhibited. Moreover, the shape of the curve for the inhibition dependance of the methylviologen reduction on the serum concentration is sigmoidal [1] which is not the case for the antiserum to polypeptide 48 000.

Table 3
Effect of antisera to different polypeptides on the light-triggered ATPase in chloroplasts from *Nicotiana tabacum*

Antiserum to polypeptides of mol. wt.	(μmoles [^{32}P]ATP-formed·(mg Chlorophyll)$^{-1}$·h^{-1})
17 000	48[a]
Control serum	48.5
% Stimulation	0
40 000	53[b]
Control serum	35
% Stimulation	51
62 000	102[a]
Control serum	53
% Stimulation	93

Chloroplasts for the reaction were prepared from *N. tabacum* var. John William's Broadleaf. [a] and [b] are results from different series of chloroplast preparations.

The antiserum to polypeptide 17 000 inhibits very slightly and in a not reproducible manner the photoreduction of methylviologen with DPiP/ascorbate as the electron donors. A low inhibition was occasionally also observed for PMS-mediated cyclic photophosphorylation. All other reactions tested such as the ferricyanide Hill reaction and photophosphorylation reactions were not influenced by the antiserum. Thus, the antiserum behaves very similar to the recently described antiserum to polypeptide 24 000 [1]. Antisera to more purified preparations of polypeptide 24 000 do not affect any electron transport reaction.

Concerning the purity of our polypeptide fractions, our earlier assumption has been confirmed that the fractions even though of uniform mol. wts. contain more than one polypeptide species. In some cases the mol. wts. differ only very slightly. In addition, we have observed that dodecyl sulfate in phosphate buffer at pH 7.2 does not separate certain reduced thylakoid proteins into their individual polypeptides. This can be demonstrated, because in alkaline medium with the same dodecyl sulfate concentration originally uniform components can be separated into peptides of very different sizes. Thus, the recently described polypeptide 66 000 consists of a mixture of molecules with more than one chain and of polypeptides with the apparent mol. wt. 66 000. The same appears to apply for other fractions. Moreover, several polypeptide fractions which in the polyacrylamide gel electrophoresis gave only one band, show in the double diffusion test against the mixture of all polypeptides

common precipitation bands. These fractions, therefore, contain despite their very different mol. wts. at least one common identical polypeptide. On the other hand it is surprising that antisera to the fractions differ so specifically in their action on photosynthetic electron transport.

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